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Characterization and Comparative Analysis of the Genes Encoding *Haemophilus parasuis* Outer Membrane Proteins P2 and P5▽†

Michael A. Mullins,[1] Karen B. Register,[1]* Darrell O. Bayles,[2] Crystal L. Loving,[1] Tracy L. Nicholson,[1] Susan L. Brockmeier,[1] David W. Dyer,[3] and Gregory J. Phillips[4]

Respiratory Diseases of Livestock Research Unit[1] and Bacterial Diseases of Livestock Research Unit,[2] USDA Agricultural Research Service National Animal Disease Center, P.O. Box 70, Ames, Iowa 50010; Microbial Pathogenesis and Microbial Genomics, University of Oklahoma Health Sciences Center Biomedical Research Center, Oklahoma City, Oklahoma 73104[3]; and Department of Veterinary Microbiology and Preventive Medicine, Iowa State University College of Veterinary Medicine, Ames, Iowa 50011[4]

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Haemophilus parasuis **is a swine pathogen of significant industry concern, but little is known about how the organism causes disease. A related human pathogen, *Haemophilus influenzae*, has been better studied, and many of its virulence factors have been identified. Two of these, outer membrane proteins P2 and P5, are known to have important virulence properties. The goals of this study were to identify, analyze, and compare the genetic relatedness of orthologous genes encoding P2 and P5 proteins in a diverse group of 35 *H. parasuis* strains. Genes encoding P2 and P5 proteins were detected in all *H. parasuis* strains evaluated. The predicted amino acid sequences for both P2 and P5 proteins exhibit considerable heterogeneity, particularly in regions corresponding to predicted extracellular loops. Twenty-five variants of P2 and 17 variants of P5 were identified. The P2 proteins of seven strains were predicted to contain a highly conserved additional extracellular loop compared to the remaining strains and to *H. influenzae* P2. Antigenic-site predictions coincided with predicted extracellular loop regions of both P2 and P5. Neighbor-joining trees constructed using P2 and P5 sequences predicted divergent evolutionary histories distinct from those predicted by a multilocus sequence typing phylogeny based on partial sequencing of seven housekeeping genes. Real-time reverse transcription-PCR indicated that both genes are expressed in all of the strains.**

Haemophilus parasuis is the causative agent of Glässer's disease, characterized by polyserositis (inflammation of the serous membranes accompanied by fibrinous exudate), polyarthritis, and meningitis, with high rates of mortality and prolonged reduction in the growth rates of affected swine (37). *H. parasuis* additionally causes pneumonia in the absence of systemic disease (23) but is also often carried in the nasal passages of seemingly healthy pigs (7, 13). There are 15 characterized *H. parasuis* serotypes, and minimal cross-protection is observed between strains (19). It is not uncommon for a single herd to be colonized with several different strains, and isolates recovered from systemic sites tend to be distinct from those recovered from the lung or nasal cavity (34, 36). Disease resulting from *H. parasuis* infection is an increasing concern to pork producers, especially those operating high-health, specific-pathogen-free herds, where infection appears to cause more serious disease (24, 37).

Little is known about virulence factors specific to *H. parasuis*, and recent studies are just beginning to reveal potential components of disease-causing mechanisms. A comparison of *H. parasuis* strains suggested that certain outer membrane protein profiles may be associated with virulence (41), although none of the proteins was further characterized. A global search for *H. parasuis* virulence genes using differential-display reverse transcription (RT)-PCR revealed relatively few promising targets (14), while other investigations assessing gene expression in vivo (17), or under in vitro growth conditions designed to mimic those encountered in vivo (26, 27), identified numerous potential virulence genes, including a variety of transporters, metabolic and biosynthetic enzymes, putative cell surface proteins, and some apparent homologs of virulence genes expressed by other members of the *Pasteurellaceae*. A cell-associated neuraminidase has also been proposed to contribute to pathogenicity (22). Recently, the lipooligosaccharide of *H. parasuis* was shown to have a limited role in adhesion to newborn pig tracheal cells (5). In the same study, *H. parasuis* was shown to induce apoptosis of epithelial cells through a lipooligosaccharide-independent mechanism. Biofilm formation has been demonstrated in numerous *H. parasuis* isolates and appears to be negatively associated with systemic spread of the organism (18).

More specific observations have been made regarding virulence factors in *Haemophilus influenzae*, a human pathogen responsible for otitis media, meningitis, and pneumonia. A number of adhesins, proteases, and other proteins involved in causing disease and stimulating host immune responses have been identified (45), including two well-characterized outer membrane proteins, P2 and P5. Outer membrane protein P2, encoded by the *ompP2* gene, is an immunodominant porin with considerable antigenic heterogeneity among strains (43) that has been targeted as a potential vaccine candidate (16). As a porin, it functions both in general diffusion and specific trans-

* Corresponding author. Mailing address: Respiratory Diseases of Livestock Research Project, USDA/ARS/National Animal Disease Center, P.O. Box 70, 2300 Dayton Road, Ames, IA 50010. Phone: (515) 663-7700. Fax: (515) 663-7458. E-mail: Karen.Register@ars.usda.gov.

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TABLE 1. *H. parasuis* strains used in this study

Strain	Serovar	Isolation site	Variant (%)[a]	
			P2	P5
No. 4	1[b]	Nose	24 (78.4)	15 (89.2)
Sw140	2[b]	Nose	10 (93.6)	11 (94.3)
Sw114	3[b]	Nose	23 (79.1)	13 (94.8)
Sw124	4[b]	Nose	21 (80.6)	9 (94.3)
Nagasaki	5[b]	Meninges	2 (99.7)	1 (100)
131	6[b]	Nose	22 (81.9)	17 (89.0)
174	7[b]	Nose	14 (95.0)	10 (94.6)
C5	8[b]	Unknown	25 (79.1)	17 (89.0)
D74	9[b]	Unknown	16 (95.0)	16 (89.0)
H367	10[b]	Unknown	15 (95.8)	3 (93.8)
H465	11[b]	Trachea	20 (78.6)	14 (94.6)
H425	12[b]	Lung	15 (95.8)	7 (96.5)
84-17975	13[b]	Lung	6 (99.2)	1 (100)
84-22113	14[b]	Joint	1 (100)	2 (96.7)
84-15995	15[b]	Lung	5 (97.8)	4 (94.0)
10680	UK[c]	Lung, heart	12 (93.9)	12 (94.6)
12939	UK	Lung	19 (82.6)	12 (94.6)
15677	UK	Brain, heart	13 (98.3)	4 (94.0)
17321	UK	Brain, lung	11 (94.2)	11 (94.3)
24054	UK	Lung	13 (98.3)	4 (94.0)
28803	UK	CSF[d], lung	9 (94.7)	12 (94.6)
29814	UK	Joint, lung	12 (93.0)	12 (94.6)
29864	UK	Brain, joint	12 (93.9)	12 (94.6)
32585	UK	Lung	12 (93.9)	6 (96.2)
831541	UK	Unknown	7 (96.4)	4 (94.0)
831542	UK	Unknown	7 (96.4)	4 (94.0)
464-99	UK	Unknown	3 (99.7)	1 (100)
685-99	UK	Unknown	4 (97.8)	5 (97.0)
1050-99	UK	Unknown	3 (99.7)	1 (100)
2170B	UK	Joint	6 (99.2)	1 (100)
ATCC 19417[T]	UK	Respiratory tract	17 (94.7)	12 (94.6)
29755	5	Lung	1	1
MN-H	13	Joint, lung, CNS[e]	18 (93.9)	8 (94.3)
NADC1	UK	Lung	8 (93.9)	7 (96.5)
NADC2	UK	Lung	1 (100)	1 (100)

[a] The value in parentheses is the percent amino acid identity compared to strain 29755.
[b] Serovar reference strain.
[c] UK, unknown.
[d] CSF, cerebrospinal fluid.
[e] CNS, central nervous system.

port of nicotinamide-derived nucleotide substrates (1). Amino acid changes affect P2 pore permeability and the effectiveness of antibiotics (39). P2 may play a role in colonization and has been shown to bind to specific components of human mucin (38). Evidence for expression of P2 in *H. parasuis* has yet to be presented, but recent genome-sequencing efforts have revealed intact *ompP2* orthologs in two different strains (49; GenBank accession no. NZ_ABKM00000000).

The P5 outer membrane protein of *H. influenzae*, encoded by the *ompP5* gene, is a member of the OmpA family and is present as a major structural protein in many gram-negative bacteria (8). P5 has both immunodominant and host-adhesive domains (31) and has been shown to bind to human mucin (38), as well as to surface-expressed carcinoembryonic antigen-related cell adhesion molecule 1 (15). Structure predictions for numerous P5 sequence variants suggest the presence of eight transmembrane regions and four surface-exposed loop regions (28, 48). The predicted fourth loop region is an immunodominant but nonprotective epitope that serves to misdirect host immune responses (30). A protein reactive with an antibody that recognizes the P5 protein of *H. influenzae* has been detected in a single *H. parasuis* strain (25), and complete open reading frames orthologous to the *H. influenzae ompP5* gene have recently been identified (50; GenBank accession no. NZ_ABKM00000000).

TABLE 2. PCR primers

Target	Primer name	Sequence (5′–3′)
ompP2	HpompP2-1	AAA AAA ACA CTA GTA GCA TTA G
ompP2	HpompP2-2	CCA TAA TAC ACG TAA ACC AA
ompP2	HpompP2-3	ATG TAA GAT ATT GAC ACT ACT CTA C
ompP2	HpompP2-4	TGA TAG AAA TAC CGA AAC GAG AAC
ompP2	HpompP2-5	CAG CTC ATC AAT TCA TGT TAG GTG CAG
ompP2	HpompP2-6	AAC GAG CCG AAT TGG AAA CCA ACG
ompP5	HpompP5-1	AAA AAA TCT TTA ATT GCA TT
ompP5	HpompP5-2	CAT AGA AAC TTC TTT TGA AC
ompP5	HpompP5-3	AAA TTT CAG CCT CGA CAC GGC TTC
ompP5	HpompP5-4	AGC GCT AGT TGC ATA ACG AGC ATC
ompP5	HpompP5-8	AAA GAT GGG CCT CAA CCT ACA GGA
ompP5	HpompP5-9	AGG TTA CGG TGA AGC TAA CCC AGT
16S[a]	Hpar16S-for	CGA CGA TCT CTA GCT GGT CTG A
16S[a]	Hpar16S-rev	AGG AGT CTG GAC CGT GTC TCA
ompP2[a]	HparP2-for	TCG TTT CGG TAT TTC TAT CAA ACA TAA
ompP2[a]	HparP2-rev	GAG TCT CAT AAC GAC CAA AAC CGT A
ompP5[a]	HparP5-for	CGG TTA CAC AGA CCG TAT TGG TAA
ompP5[a]	HparP5-rev	TTT CTG CAC GTT TTT GTG AAA GA

[a] Used for RT-PCR.

The goals of the current study were to identify orthologs of the genes encoding P2 and P5 from a diverse group of *H. parasuis* isolates, including representatives of the 15 described serovars and several field isolates, and to examine and compare their predicted structural and antigenic properties. Expression of *ompP2* and *ompP5* based on detection of mRNA was also evaluated for all strains. To determine whether unique selective pressures might drive the emergence of P2 or P5 sequence variants, an evolutionary phylogeny of strains based on DNA sequences derived from housekeeping genes was compared with trees constructed on the basis of the *ompP2* or *ompP5* sequence.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. Thirty-five strains of *H. parasuis* were selected for study, including field strains obtained in the United States, Europe, and Japan; the 15 recognized serovar reference strains (19); and the ATCC type strain. A list of the strains used is presented in Table 1. The strains were grown on Casman Agar Base (Becton Dickinson, Sparks, MD) supplemented with 1% (wt/vol) NAD (Sigma, St. Louis, MO) and 5% Gibco filtered horse serum (Invitrogen, Carlsbad, CA) at 37°C under 5% CO_2.

Southern blotting. Genomic DNA was prepared using a commercially available purification kit (Promega, Madison, WI) and quantified using UV spectrophotometry. Three micrograms of genomic DNA was digested overnight at 37°C with restriction endonuclease NciI (Invitrogen, Carlsbad, CA), whose recognition site is not found within any known *H. influenzae ompP2* or *ompP5* sequence. Fragments were resolved by electrophoresis overnight in 0.6% agarose gels containing 0.5 μg of ethidium bromide per ml in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM disodium EDTA) and transferred to positively charged nylon membranes (Roche Applied Science, Indianapolis, IN). DNA was bound to membranes using a UV transilluminator.

The high-stringency conditions used for membrane hybridization and processing were previously reported (40). *ompP2* and *ompP5* probes were digoxigenin (DIG) labeled with a PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations, using *H. parasuis* strain 29755 DNA as the template. Primers HpompP2-1 and HpompP2-2 were used to generate the *ompP2* probe; primers HpompP5-1 and HpompP5-2 were used to generate the *ompP5* probe (Table 2). Detection with anti-DIG–alkaline phosphatase and CSPD (Roche Applied Science, Indianapolis, IN) was carried out according to the manufacturer's protocol. To visualize hybridization, X-ray film (Kodak, Rochester, NY) was exposed to the membrane for 5 to 40 min and then developed.

Sequencing and analysis of the *ompP2* and *ompP5* genes. PCR primers were designed to amplify *ompP2* and *ompP5* in *H. parasuis* based on the DNA sequences of these genes in strain 29755 (Table 2). For *ompP2*, primers HpompP2-1 and HpompP2-2 amplified the open reading frame, minus the start

and stop codons (1,074 bp in 29755). Overlapping amplicons covering the 5′ and 3′ ends were obtained using primer sets HpompP2-3/HpompP2-4 and HpompP2-5/HpompP2-6, respectively. A similar approach was utilized for *ompP5* with primer sets HpompP5-1/HpompP5-2, HpompP5-3/HpompP5-4, and HpompP5-9/HpompP5-8.

PCR was performed on a PTC-200 thermocycler (MJ Research, Waltham, MA) using the following cycling conditions: 95°C for 5 min, 35 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 1 min, and a final elongation of 72°C for 7 min. Reaction mixtures contained a final concentration of 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 2.5 U of *Taq* polymerase, and 0.5 μM of each primer in a final volume of 50 μl. Following confirmation of a single PCR product by agarose gel electrophoresis, amplicons were purified using spin columns (Qiagen, Valencia, CA) and sequenced directly by fluorescence-based cycle sequencing with AmpliTaq and BigDye Terminators on an ABI 377 sequencer at the National Animal Disease Center Genomics Unit. PCR products from a subset of strains were cloned into pCR2.1 using a TA Cloning Kit (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Recombinants selected using blue-white screening with 5-bromo-4-chloro-3-indolyl-β-D-galactoside and isopropylthiogalactoside on Luria-Bertani agar plates supplemented with 50 μg/ml kanamycin were picked directly into PCR tubes, and the inserts were amplified and sequenced as indicated above. All sequences were edited and aligned using the Vector NTI Advance 10 program Contig Express (Invitrogen, Carlsbad, CA). The final sequences represent a minimum of threefold coverage with at least one read from each strand.

DNA sequences were translated in silico into protein sequences and analyzed as follows. The Compute pI/M_w tool (http://www.expasy.ch/tools/pi_tool.html) was used to compute the molecular weights of P2 and P5. PSORT (29) was used to predict the subcellular locations of the two proteins. InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) was used to assess the functional role of each protein. Hidden Markov modeling and neural network modeling in SignalP (http://www.cbs.dtu.dk/services/SignalP/) were both used to detect signal peptides and predict the cleavage sites within each sequence. The PRED-TMBB β-barrel prediction tool (2) was used to describe the structures of proteins in their final cellular locations and to generate two-dimensional visual representations of the proteins within the membrane. Antigenic sites were detected using a semiempirical prediction method (21). Simpson's index of diversity (44) was calculated for P2 and P5 variants as a measure of genetic diversity. The values obtained for a particular set of sequences can range from 0 (every sequence identical) to 1.0 (every sequence unique).

***ompP2* and *ompP5* expression.** Real-time RT-PCR was used to detect *ompP2*- and *ompP5*-specific mRNAs. Bacteria were grown to an A_{600} of 0.3 ± 0.05, pelleted by centrifugation, and resuspended in RLT buffer for RNA isolation using the RNeasy Mini Isolation Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. cDNA synthesis was completed using random primers and SuperScript II Reverse Transcriptase according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). For real-time PCR, gene-specific primers (Table 2) were designed from conserved regions evident in multiple-sequence alignments using Primer Express software (Applied Biosystems, Foster City, CA), yielding amplicons of 68 and 65 nucleotides from *ompP2* and *ompP5*, respectively. Primers specific for the *H. parasuis* 16S rRNA gene (Table 2) were used for positive control reactions. Negative control reactions using isolated RNA not reverse transcribed into cDNA were also performed to verify that amplification of chromosomal DNA did not contribute to the results obtained.

MLST. Multilocus sequence typing (MLST) was carried out following the methods described by Olvera et al. (33). Using the primer sequences provided therein, PCR amplicons were obtained from seven *H. parasuis* housekeeping genes: *atpD*, *infB*, *mdh*, *rpoB*, *6pgd*, *g3pd*, and *frdB*. The amplicons were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) following confirmation of a single PCR product by agarose gel electrophoresis. Sequencing and analysis were carried out as described above. The final sequences represent a minimum of threefold coverage with at least one read from each strand. The final sequences derived from each gene for all strains were aligned and trimmed to equal lengths based on the outermost 5′- and 3′-terminal positions available for every strain. The resulting sequence lengths were 548 bp for *atpD*, 441 bp for *infB*, 431 bp for *mdh*, 366 bp for *rpoB*, 416 bp for *6pgd*, 400 bp for *g3pd*, and 450 bp for *frdB*. These sequences are slightly truncated at both ends compared to those reported previously for MLST analysis of *H. parasuis* (33). MLST data for 11 of the 35 strains evaluated in this study have been reported previously (33).

Phylogenetic analyses. Neighbor-joining trees used to infer the evolutionary history of *ompP2*, *ompP5*, or MLST DNA sequences were constructed using MEGA4 (42, 46). The evolutionary distances were computed using the maximum composite likelihood method (47), and the units are the number of base substitutions per site. For the MLST tree, sequences from the seven housekeeping genes *atpD*, *infB*, *mdh*, *rpoB*, *6pgd*, *g3pd*, and *frdB* were concatenated, in that order, into a single sequence of 3,052 bp for each *H. parasuis* strain prior to phylogenetic analysis. Bootstrap consensus trees inferred from 10,000 replicates were taken to represent the evolutionary history of the taxa analyzed (11). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. All positions containing gaps and missing data were eliminated from data sets (complete deletion) used for unrooted trees. For trees rooted using sequences from *H. influenzae* strain Rd KW20, positions containing alignment gaps and missing data were eliminated only as needed in pairwise sequence comparisons (pairwise deletion).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *ompP2* and *ompP5* sequences reported here are EU741863 to EU741929 and EU852097 to EU852099.

RESULTS

Identification of *ompP2* and *ompP5* genes and comparative DNA sequence analysis. *ompP2*- and *ompP5*-specific PCR demonstrated that both genes are present in all 35 strains of *H. parasuis* evaluated. DNA sequences of PCR amplicons obtained from strain 29755 are identical to those obtained previously from the draft genome sequence. DNA sequence alignments revealed considerable heterogeneity among strains in both genes. The open reading frame (ORF) for *ompP2* in strain 29755 is 1,080 bp and ranges in length from 1,077 to 1,203 bp for the remaining strains. Among all strains, 25 different *ompP2* alleles were observed, with no more than four strains sharing any single variant (Table 1). Relative to strain 29755, two large, in-frame inserts 48 to 60 bp and 67 to 70 bp in length are present in seven strains (no. 4, Sw114, Sw124, 131, C5, H465, and 12939). Slightly less heterogeneity was observed among *H. parasuis ompP5* genes. The *ompP5* ORF is 1,104 bp in strain 29755 and varies in length from 1,098 to 1,110 bp in all other strains. Seventeen unique *ompP5* alleles were identified, distinguished by substitutions and/or small (3- to 12-bp) insertions or deletions. As many as seven strains were found to share a single allelic variant. Considering individual isolates, 28 different *ompP2-ompP5* allelic combinations were identified. Four of the seven *ompP2* alleles occurring in more than a single isolate are associated with only a single *ompP5* variant, although the suggested exclusivity of the relationships may be an artifact of the limited number of strains representing each *ompP2* allele. Conversely, none of the six *ompP5* alleles common to more than one isolate are associated solely with a particular *ompP2* variant.

Based on available *H. parasuis* genome sequence data, *ompP2*- and *ompP5*-containing NciI fragments are predicted to be approximately 5.1 kb and 6.4 kb, respectively. Although no evidence currently exists for multiple copies of either *ompP2* or *ompP5* in *H. influenzae*, Southern blotting was used to examine the copy numbers of these genes in the *H. parasuis* strains included in this study. An *ompP2* probe hybridized with a single fragment of approximately 5.1 kb in all 35 of the strains evaluated, but unexpectedly, an additional strongly hybridizing band of roughly 10 kb was apparent in 4 strains (results from representative strains are shown in Fig. 1, left). To address the possibility that incomplete DNA digestion might explain these results, the blots were stripped and rehybridized with a probe

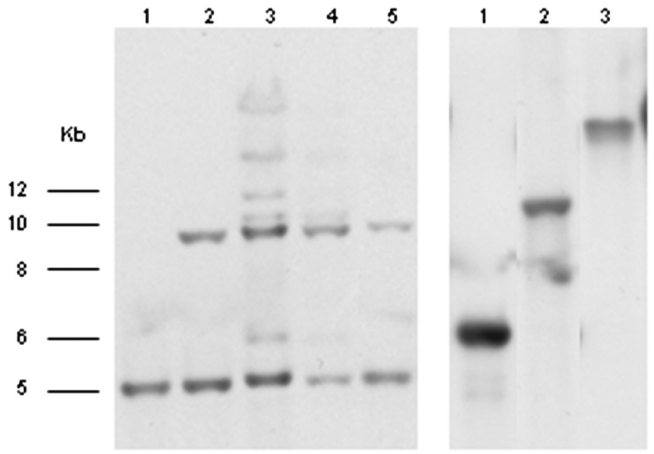

FIG. 1. Southern blots of NciI-digested *H. parasuis* genomic DNA from the strains indicated hybridized to an *ompP2* (left) or *ompA* (right) probe. (Left) Lanes: 1, H425; 2, 831542; 3, 24054; 4, 15677; 5, Nagasaki. (Right) Lanes: 1, 831542; 2, 17321; 3, 15677. The relative positions of the DNA size markers are indicated to the left.

specific for an unrelated capsular biosynthesis gene expected to be present in a single copy. Only one band was evident in every strain (data not shown). When the blots were hybridized with an *ompP5* probe, a single fragment of the size predicted (6.4 kb) was detected in 27 strains, including 29755; single fragments of either approximately 11.5 kb or 20 kb were detected in the remaining strains, suggesting either that *ompP5* is located in a different region of the chromosome in these strains or that the location of the flanking NciI sites is altered. Figure 1, right, includes a strain representative of each of the different fragments observed.

Given the highly stringent hybridization conditions used, these results suggest that multiple copies of *ompP2* are present in some strains of *H. parasuis*, raising the question of whether multiple allelic variants might occur within a single strain. Reexamination of the trace data from the previously sequenced *ompP2* PCR products failed to identify potentially polymorphic sites for any strain that displayed multiple *ompP2*-containing bands by Southern blotting. Additionally, the DNA sequences of 10 individually cloned amplicons from each of the strains in question revealed no variation within any single strain.

P2 and P5 amino acid and structural predictions. All allelic variants described for both genes are predicted to give rise to unique protein sequences (Fig. 2). No frameshift or nonsense mutations were detected in either gene for any strain. Depending on the strain, the calculated molecular masses of the mature P2 and P5 proteins (i.e., missing the signal sequences) range from 36.7 to 40.1 kDa and 37.1 to 37.4 kDa, respectively.

Compared to strain 29755, the relative amino acid identity for P2 ranges from 78.4 to 100%, while that of P5 ranges from 89.0 to 100% (Table 1). The degree of diversity among all 35 *H. parasuis* P2 sequences is greater than the level found among P5 sequences (Simpson's index of diversity, 0.976 versus 0.918, respectively). For comparative purposes, diversity indices were also calculated using the P2 and P5 sequences of *H. influenzae* currently available in GenBank. In contrast to *H. parasuis*, less diversity was apparent among *H. influenzae* P2 proteins (an index of 0.994, representing 57 sequences) than among P5 proteins (an index of 1.0, representing 13 sequences). Taking sample size into consideration, a higher level of variation appears to exist among P5 proteins of *H. influenzae* than among those of *H. parasuis*.

Unique pairings of P2 and P5 proteins occur in 22 of the 35 strains examined (Table 1). Six combinations are represented among the 13 strains whose P2/P5 pairings are not unique; five are common to 2 strains each, and one is shared by 3 strains. In some cases, strain designations suggest that common pairings might merely reflect different isolations from the same herd or outbreak, (e.g., strain pairs 831541/831542 and 464-99/1050-99), but this is conjecture, since no information is available regarding the epidemiologic relationship among these strains or most of the others evaluated here. Of note, the three strains sharing P2/P5 variant 12/12 appear to be distinct from one another, based on MLST (see below and Fig. 5C) and were all obtained from pigs with septicemia. While intriguing, the absence of additional information related to the source of these strains prevents any sound conclusion as to the significance of this association or the potential contribution of this P2/P5 pairing to disease manifestation or virulence.

PSORT predicted that both P2 and P5 function as outer membrane proteins. InterProScan predicted that all P2 proteins are outer membrane proteins of the gram-negative porin family and that all P5 proteins are outer membrane proteins of the OmpA family. Signal P hidden Markov modeling and neural network modeling detected similar amino-terminal signal sequences in both proteins: in P2, a signal peptide is predicted to be cleaved at position 20 in all strains, and in P5, a signal peptide is predicted to be cleaved at position 26 in all strains.

PRED-TMBB β-barrel prediction analysis was used to approximate the final conformation and two-dimensional arrangement of the *H. parasuis* P2 and P5 proteins in the bacterial outer membrane. For the P2 protein, two distinct forms were predicted. The first, exhibited by 28 of the 35 strains examined, consists of an outer membrane protein with eight extracellular-loop domains, numbered consecutively as loops 1 to 8 (Fig. 2A). Figure 3, left, depicts the structural representation of P2 from strain 15677, whose predicted sequence most closely approximates the consensus sequence. The predicted structure of the *H. influenzae* P2 protein similarly includes eight loops (Fig. 3, right). The P2 proteins of the seven remaining *H. parasuis* strains examined possess two large insertions relative to the consensus sequence, one contained entirely within loop 3 and the other within loop 5 (Fig. 2A). For these strains, PRED-TMBB predicted a ninth external loop; the P2 structure from a representative strain (H465) is depicted in Fig. 3, middle. Specifically, a 15- to 20-amino-acid insertion in loop 3, as defined in the consensus sequence, creates an additional transmembrane region predicted to split the loop into two shorter extracellular regions, loops 3a and 3b. The second insertion of 22 to 23 amino acids is predicted to extend the transmembrane segment between loops 4 and 5 so that the N-terminal boundary of the extracellularly exposed sequence of loop 5 is shifted toward the C terminus (from DDYKSGSVNKKDK to VEAGGKSTDHTYTEKPF). For all P2 proteins, sequence heterogeneity occurs primarily within predicted extracellular-loop domains, whereas sequence outside those domains is highly conserved across strains.

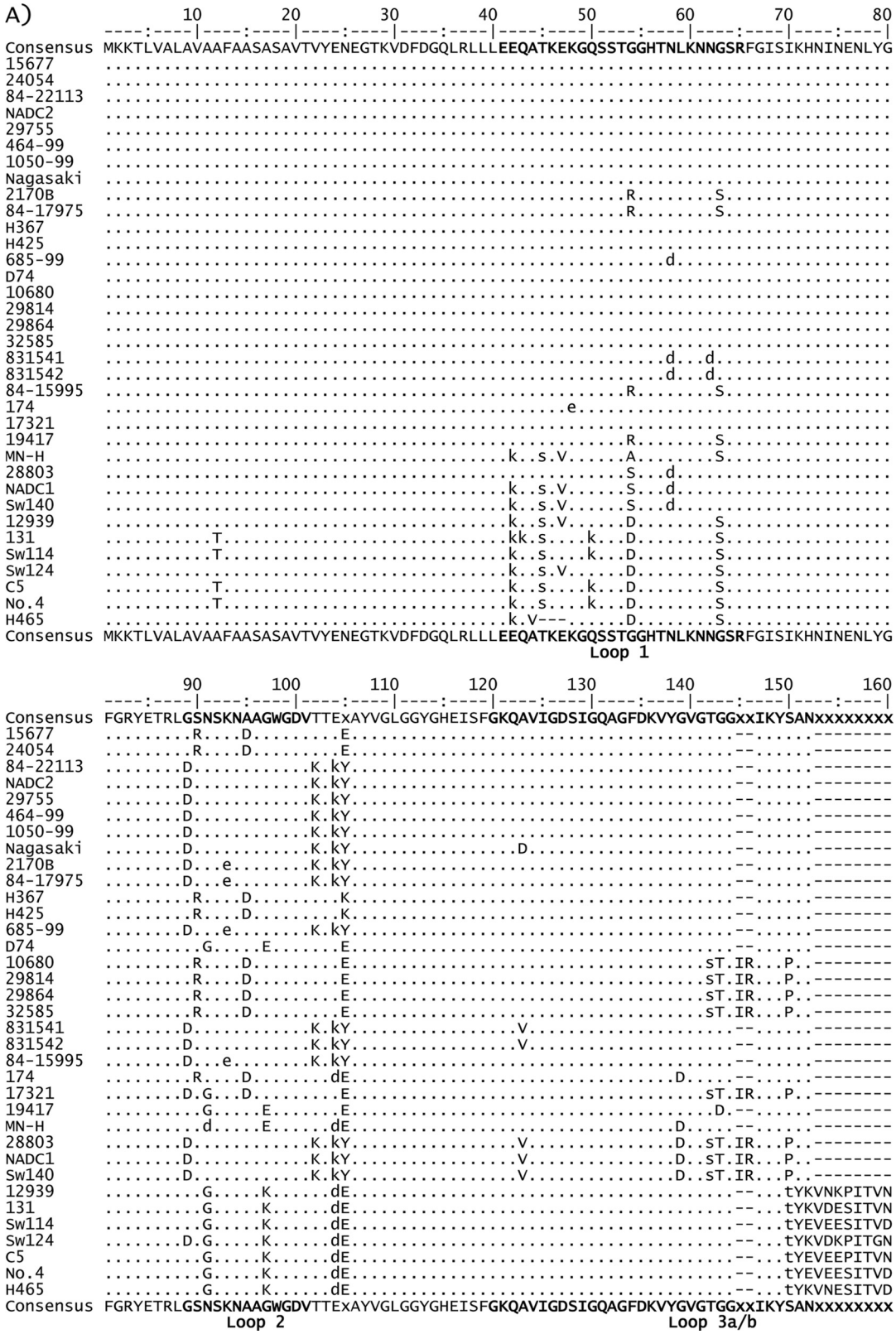

FIG. 2. Alignment of *H. parasuis* P2 (A) and P5 (B) predicted protein sequences. Predicted extracellular-loop domains are indicated in boldface within the consensus sequence. Dots represent amino acid identity with the consensus sequence, and dashes represent gaps. Lowercase letters indicate that the corresponding substituted amino acid and the consensus amino acid have similar physical properties; uppercase letters indicate an amino acid substitution with dissimilar physical properties. An "x" in the consensus sequence indicates an amino acid present in some strains that is not in the consensus.

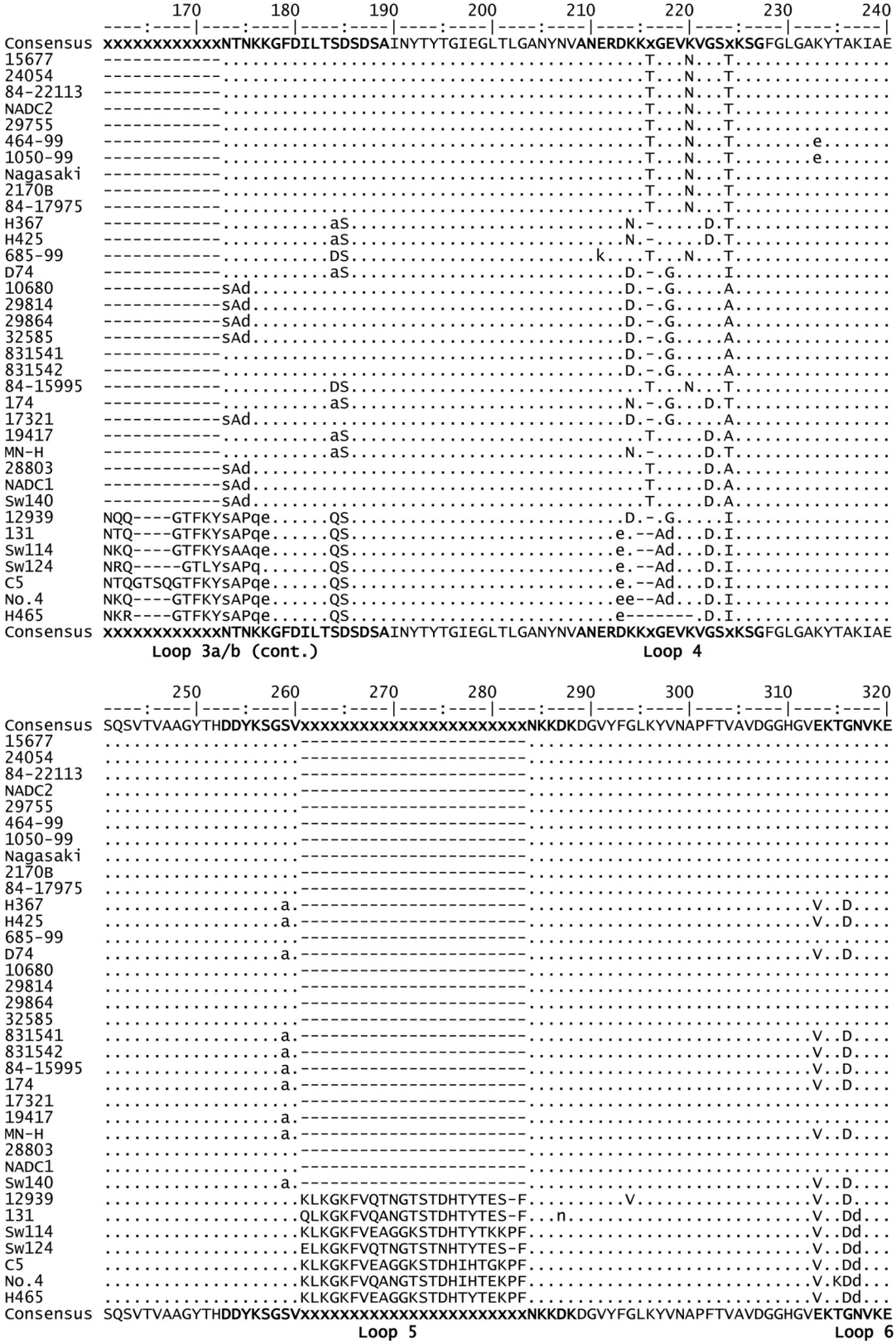

FIG. 2—*Continued.*

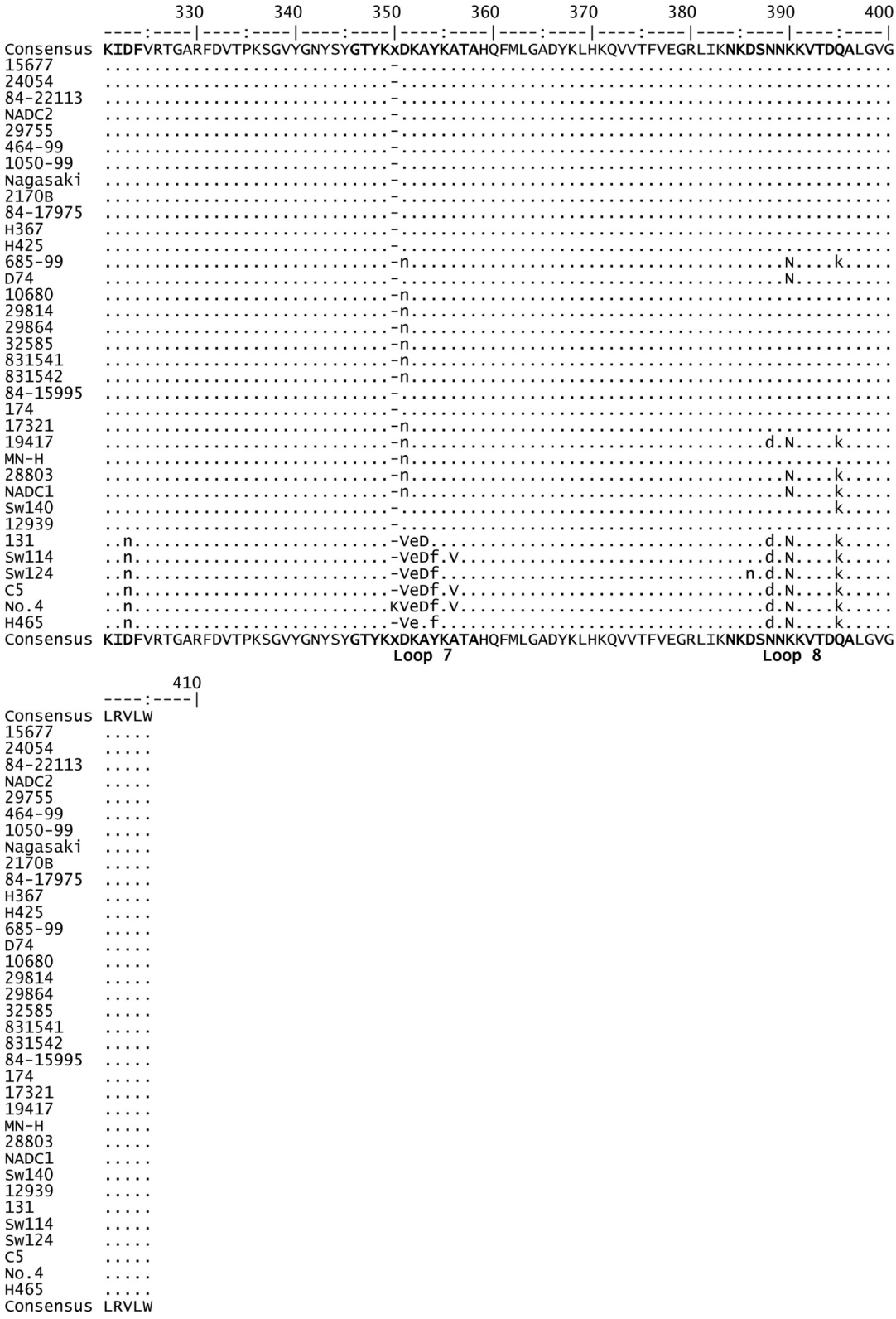

FIG. 2—*Continued.*

For P5, PRED-TMBB predicted five extracellular-loop domains, consecutively numbered 1 to 5, in all 35 *H. parasuis* strains (Fig. 2B). The P5 structure within the bacterial cell membrane for NADC1 and D74, the strains most and least similar to the consensus sequence, respectively, are depicted in Fig. 4, left and middle. Similarly to P2, sequence heterogeneity in P5 is primarily localized to predicted extracellular loop domains, and relative to the consensus sequence, insertions or

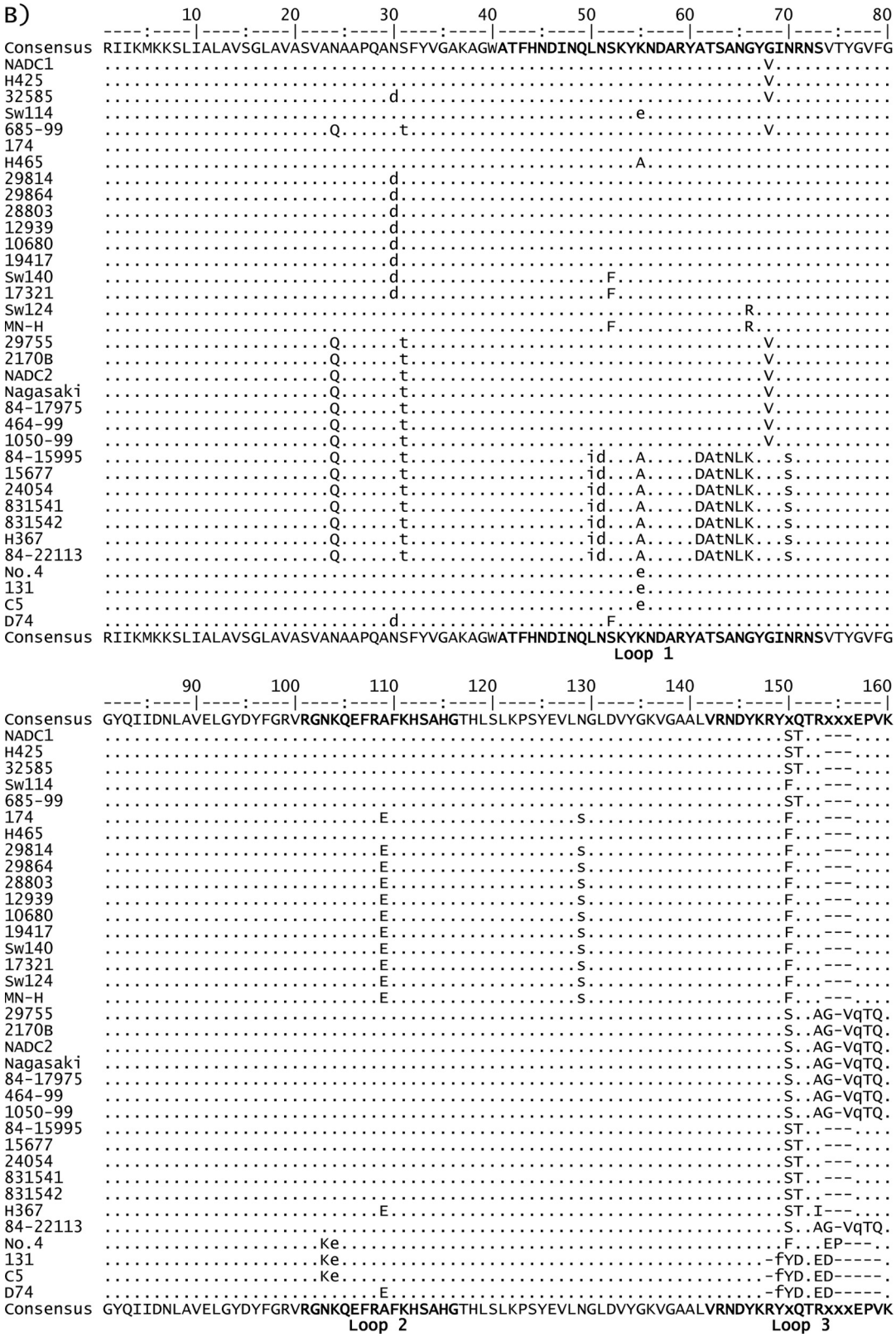

FIG. 2—*Continued.*

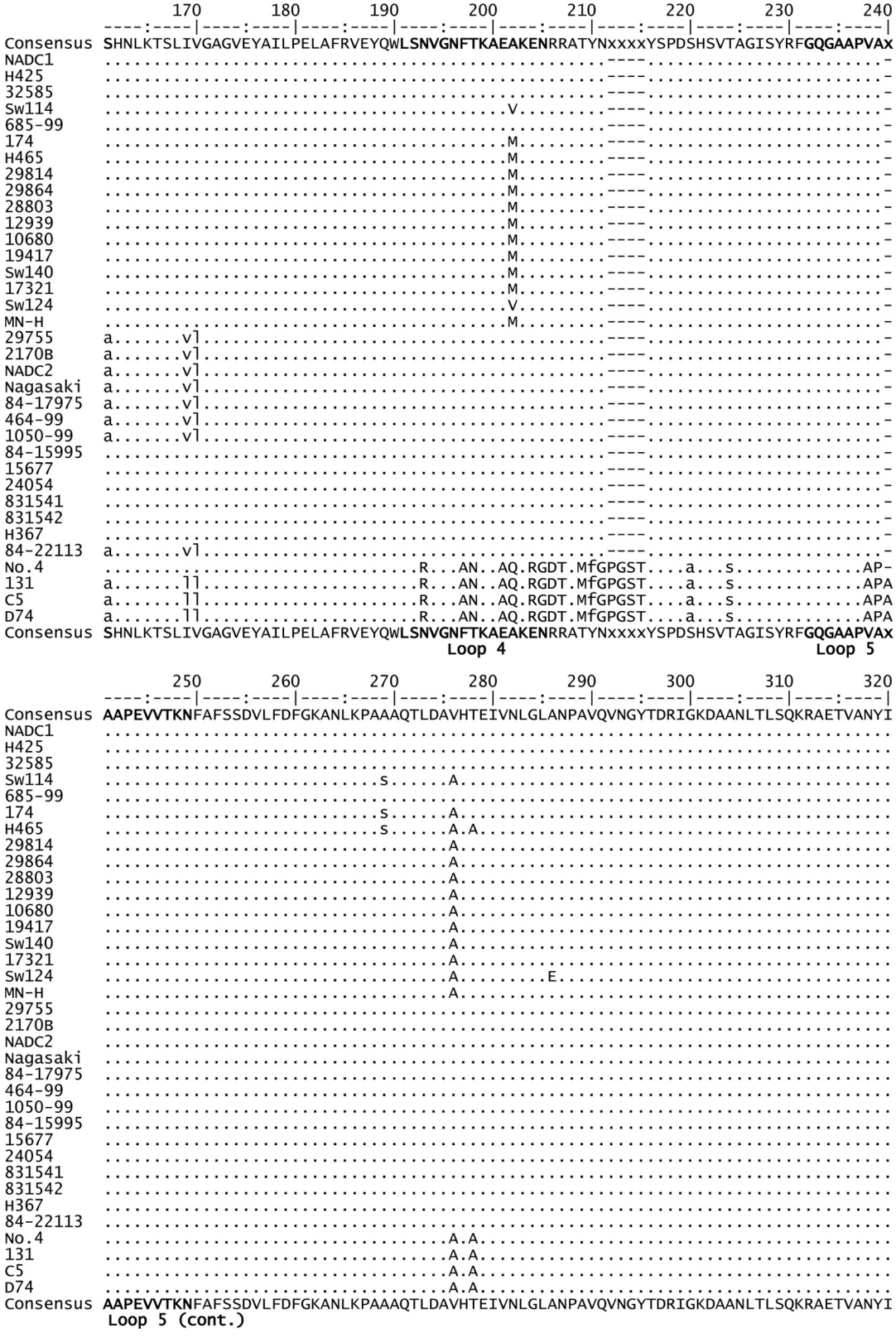

FIG. 2—*Continued.*

```
                330       340       350       360       370       380
           ----:----|----:----|----:----|----:----|----:----|----:----|
Consensus  VSKGVNPANVTAVGYGEANPVTGNTCDAVKGRKALITCLAPDRRVEIQVQGSKEVSM
NADC1      ..................................................
H425       ..................................................
32585      ..................................................
Sw114      ..................................V...............
685-99     ..................................................
174        ..................................V...............
H465       ..................................V...............
29814      ..................................V...............
29864      ..................................V...............
28803      ..................................V...............
12939      ..................................V...............
10680      ..................................V...............
19417      ..................................V...............
Sw140      ..................................V...............
17321      ..................................V...............
Sw124      ..................................V...............
MN-H       ..................................V...............
29755      ..................................................
2170B      ..................................................
NADC2      ..................................................
Nagasaki   ..................................................
84-17975   ..................................................
464-99     ..................................................
1050-99    ..................................................
84-15995   ..................................................
15677      ..................................................
24054      ..................................................
831541     ..................................................
831542     ..................................................
H367       ..................................................
84-22113   ..................................................
No.4       ..................................V...............
131        ..................................V...............
C5         ..................................V...............
D74        ..................................V...............
Consensus  VSKGVNPANVTAVGYGEANPVTGNTCDAVKGRKALITCLAPDRRVEIQVQGSKEVSM
```

FIG. 2—*Continued.*

deletions have effects on the predicted extracellular loop domain sequence and structure. Loop 3 sequences are highly variable across strains, with six variants exhibiting multiple insertions or deletions relative to the consensus. In comparison, loops 2 and 5 are more highly conserved, with only a few amino acid substitutions and, for strains 131, C5, and D74, insertion of an alanine residue in the middle of loop 5. PRED-TMBB also predicted five loops for the P5 protein of *H. influenzae* (Fig. 4, right). While modeling based on alternative methods generally suggests only four surface-exposed regions in this protein (28, 48), at least one previous study revealed areas of heterogeneity at amino acid positions consistent with a possible fifth loop (9).

All predicted *H. parasuis* P2 and P5 proteins were further analyzed for antigenic regions using a semiempirical prediction method (21). The regions identified were then compared with regions predicted to comprise extracellular loops in order to assess potential loop antigenicity. A strong hit was defined as a predicted antigenic site occurring solely within an extracellular loop region; a weak hit was defined as a site that primarily

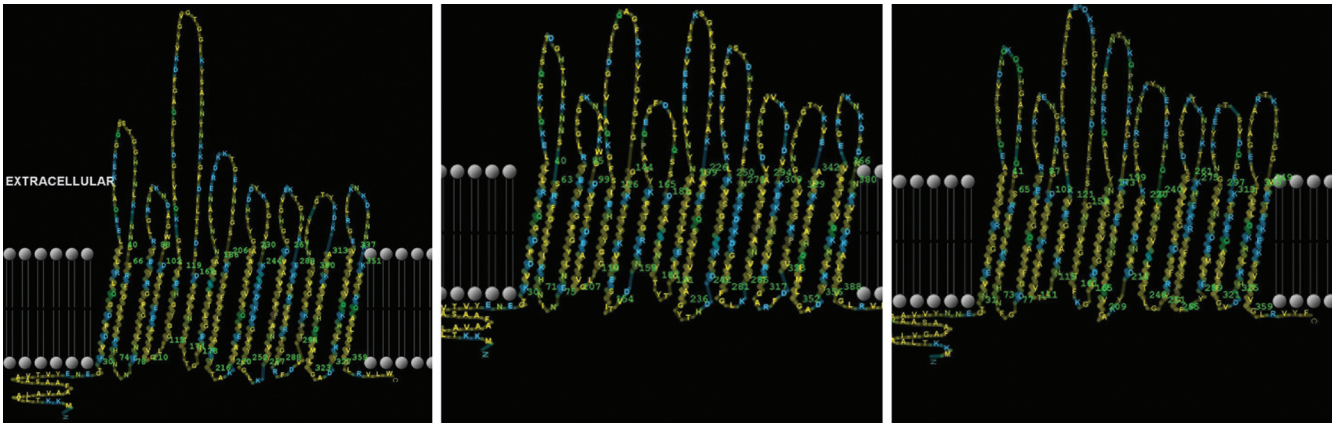

FIG. 3. P2 protein structural representations for *H. parasuis* strain 15677, the strain with greatest similarity to the consensus sequence (left); *H. parasuis* strain H465, the strain with least similarity to the consensus sequence (middle); and, for comparison, *H. influenzae* strain Rd KW20 (right). Amino acid residues are colored according to hydrophobic potential with gradation from yellow (0.5) to blue (−2.0).

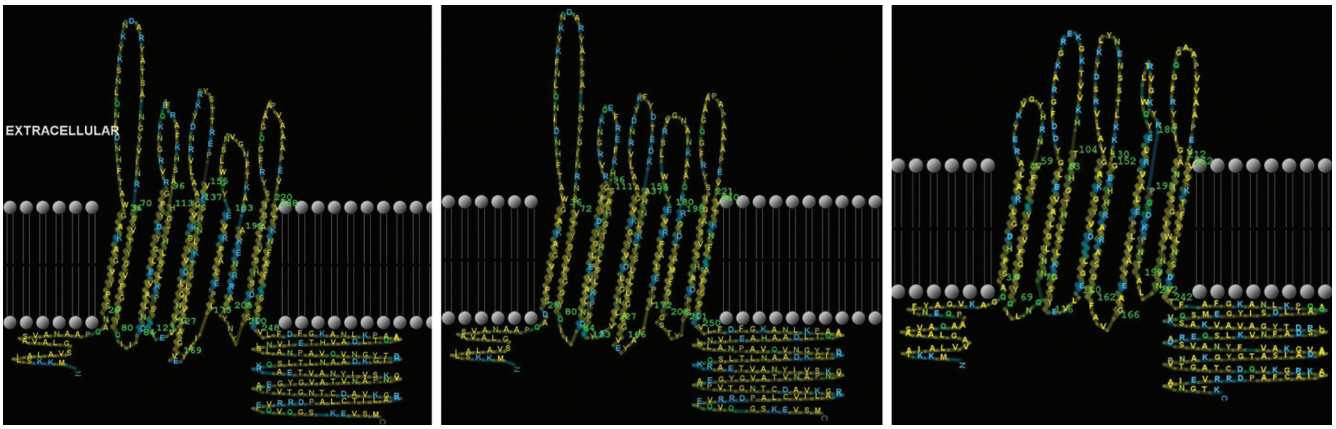

FIG. 4. P5 protein structural representations for *H. parasuis* strain NADC1, the strain with greatest similarity to the consensus sequence (left); *H. parasuis* strain D74, the strain with least similarity to the consensus sequence (middle); and, for comparison, *H. influenzae* strain Rd KW20 (right). Amino acid residues are colored according to hydrophobic potential with gradation from yellow (0.5) to blue (−2.0).

spans a transmembrane or periplasmic region, which is less likely to be antigenic, but which also includes at least some amino acids from an adjacent extracellular-loop region.

For P2, loops 1, 4, 6, 7, and 8 possess weakly antigenic motifs. In all 35 strains, loop 3 exhibits particularly high antigenicity, with multiple strongly antigenic sites predicted. In strain 15677, for example, in which loop 3 spans amino acids 120 to 167, three strongly antigenic surface-exposed sites are predicted, at positions 120 to 127, 134 to 140, and 157 to 162. Three antigenic sites were also predicted within loops 3a/3b for the P2 proteins of the seven strains with altered loop 3 morphology, but only two were scored as strong hits; in these strains, the second antigenic site of loop 3 is located within the transmembrane space, rather than extracellularly, due to the additional transmembrane region that splits loop 3 into separate extracellular domains 3a and 3b.

For P5, only weakly antigenic sites were predicted in loops 1 to 4. Loop 5, however, appears strongly antigenic in all strains. In strain NADC1, loop 5 was predicted to span amino acids 225 to 241, and a strong hit was predicted at amino acids 227 to 241. In strain D74, loop 5 was predicted to span amino acids 226 to 243, and a strong hit was predicted at 228 to 243.

***ompP2* and *ompP5* expression.** Using RT-PCR, mRNA for both P2 and P5 was detected in all 35 strains of *H. parasuis* evaluated (data not shown). These results suggest that the respective proteins are likely to be expressed by all strains, but development of antibodies for their detection is required for definitive demonstration of the presence of these proteins.

***ompP2*, *ompP5*, and MLST phylogenies.** Nucleic acid coding sequences for *ompP2* and *ompP5* were used to construct separate neighbor-joining trees (Fig. 5A and B, respectively). Although two major evolutionary lineages are apparent for both genes, and some strains segregate similarly, the phylogenies are largely divergent. This observation could indicate that different selective pressures and functional constraints are driving the emergence of P2 and P5 variants, or on a more holistic level, it may reflect differential responses to the sum of all selective pressures encountered in the particular niche(s) inhabited by *H. parasuis*.

For comparative purposes, each tree was also rooted with the appropriate *H. influenzae* ortholog (see Fig. S1 in the supplemental material), and in both cases, the degree of interspecies divergence within *H. parasuis* is minimal in comparison to intraspecies divergence.

From the 35 *H. parasuis* strains examined, 32 MLST sequence types were identified, some unique relative to those previously described for *H. parasuis* (unpublished data). Not considering short stretches of sequence missing from the 5′ and 3′ ends, which were trimmed to satisfy our coverage requirements, allelic sequences derived in this study from the 11 *H. parasuis* strains that were also included in the previous report of Olvera et al. (33) are identical to theirs, except in the case of D74, for which six of seven alleles differ. Accidental cross-contamination with another isolate in our laboratory can be eliminated as an explanation for this discrepancy, since both *ompP2* and *ompP5* sequences from our culture of D74, as well its MLST sequence type, are different from those of all other isolates in our collection. Regardless of the explanation, our seed stock of strain D74 clearly differs from that used in the study of Olvera et al. (33) and, accordingly, will henceforth be referred to as NADC-D74.

The population structure of the *H. parasuis* strains examined here, as revealed by a neighbor-joining tree based on MLST data (Fig. 5C), is distinct from the phylogenies suggested by either *ompP2* or *ompP5* sequence data. Since the housekeeping genes from which MLST data were obtained are expected to be highly conserved, the MLST tree likely reflects the overall ancestral relationship among the strains included. The P2 and P5 phylogenies are far more likely to reflect local environmental selective pressures, as evidenced by the extensive degree of diversity that is localized to the protein regions predicted to be exposed on the cell surface.

Experimental data related to virulence in swine exists only for a small number of the *H. parasuis* strains used here (19). Examination of the previously derived P2, P5, and MLST phylogenies in light of this information does not reveal any clonal relationships shared specifically among either virulent or avirulent strains. Anecdotal information related to virulence is also available for some strains, but again, no virulence-specific association with particular clones of any phylogeny is apparent.

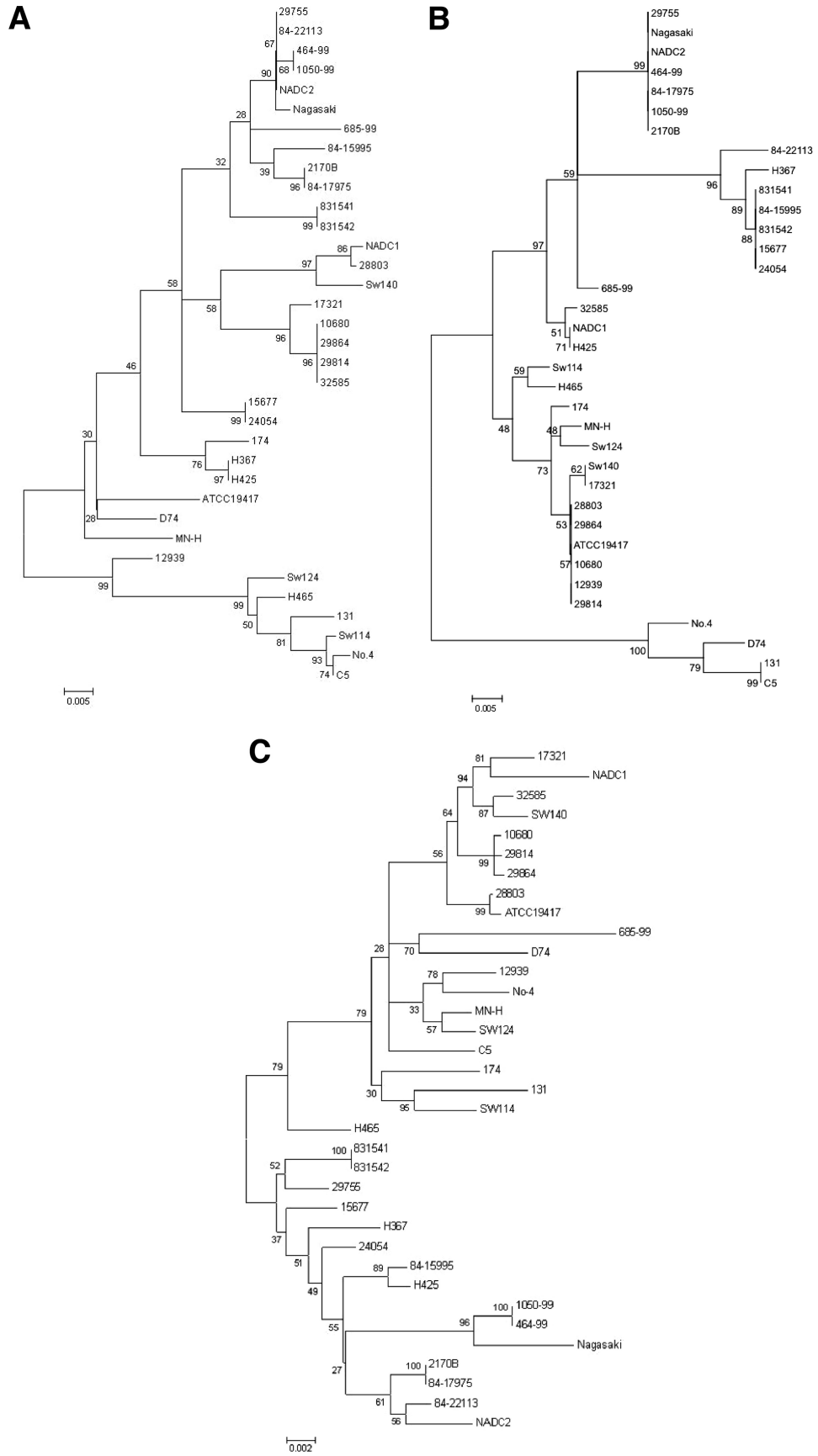

FIG. 5. Unrooted neighbor-joining trees derived from *H. parasuis ompP2* (A), *ompA* (B), and MLST (C) sequences. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches.

Similarly, considering strains for which the information is available, there is no strong correlation between the site of isolation and any major clade of either P2, P5, or MLST trees.

DISCUSSION

Previous research on the human pathogen *H. influenzae* indicates that outer membrane proteins P2 and P5 have important roles in infection and evasion of host immunity. In this report, we identified and characterized the genes encoding these proteins in 35 strains of *H. parasuis*, an economically important swine pathogen. Based on their predicted amino acid sequences, 25 P2 and 17 P5 types were detected among the strains included in this study. For P2, no more than four strains share the same predicted amino acid sequence. Although P5 sequences exhibit less overall diversity, no more than seven strains share the same variant. The degree of P2- and P5-specific heterogeneity evident among the relatively limited number of isolates examined suggests the existence of a large number of undiscovered variants circulating among infected swine. An additional degree of strain-specific variability is conferred by the many novel P2/P5 pairings in individual isolates. Interestingly, the predicted P2 and P5 proteins of a Chinese *H. parasuis* isolate whose complete genome sequence was recently reported (49) are different from any identified here.

The relationships among *H. parasuis* strains inferred from trees based on *ompP2* and *ompP5* differ from those based on MLST data, indicating that the evolution of P2 and P5 has occurred independently from that of housekeeping genes assumed to be under neutral selection. Heterogeneity in both genes is focused largely at the predicted extracellular loop regions presumably exposed to changing conditions in the host and environment. In *H. influenzae*, it has been proposed that antigenic drift of P2 and P5 results from immune selection of spontaneously occurring loop sequence variants (9, 10, 48). The extensive variability detected here in regions of *H. parasuis* P2 and P5 predicted to be both extracellular and antigenic is consistent with the hypothesis that antigenic variation of these proteins may also be driven by host immune selection (6). The *H. parasuis ompP2* and *ompP5* phylogenies additionally differ from one another, with the predicted P2 protein sequences exhibiting greater overall diversity than those predicted for P5, suggesting that the two proteins may be subject to different selective pressures or that P2 may tolerate a higher level of sequence variation without compromising function. In *H. influenzae*, P2 and P5 are postulated to contribute to colonization and are known to affect immune responses, but little is currently understood about the functions of these proteins in *H. parasuis*. In *H. influenzae*, predicted surface-exposed loops of P2 can activate the eukaryotic MAP kinase signaling pathway (12), which plays a key role in regulating both innate and adaptive immune responses (51). Loop 3 of the *H. influenzae* P5 protein is critical for protection, but a neighboring immunodominant loop appears to divert the immune response, functioning as a nonprotective decoy (30).

Of particular interest are the large insertions of highly conserved sequence discovered within loops 3 and 5 of the P2 protein in seven of the strains examined. These sequences provide the basis for the divergence of *H. parasuis* strains into two distinct lineages on the P2 tree (Fig. 5A). The mechanism that generates such variation is unclear, but horizontal exchange is possible, given that *H. parasuis* is naturally competent (3). While their origins and functional significance are unknown, these insertions have dramatic effects on the predicted surface-exposed regions of the proteins. In addition to substantially lengthening the P2 molecule, from approximately 360 amino acids in the majority of strains to approximately 400 residues in the seven affected strains, the additional amino acids in loop 3 are predicted to create a new transmembrane region. This new region may be of particular importance, as its addition removes from the extracellular space a site otherwise predicted to be highly antigenic. Additionally, in loop 5, an entirely different string of amino acids is shifted into the predicted loop region by the addition of extra residues. In both cases, not only the antigenicity but also the function of the protein may be affected.

All of the findings related to protein structure were derived from the use of predictive software tools and are, therefore, subject to limits. Selection of PRED-TMBB as our primary prediction tool was based on a preliminary assessment of its performance in comparison to another frequently used method, PROFtmb (4). Both programs use hidden Markov models and a selected set of known structures from the Protein Data Bank to develop their predictive processes. PRED-TMBB uses the sequences of 16 proteins with known structures as a training set; PROFtmb uses 8 protein sequences. PROFtmb was less consistent than PRED-TMBB in its predictions among *H. parasuis* strains with closely related sequences, and it predicted loops at some locations that appear biologically unrealistic based on current knowledge of the structure of transmembrane β-barrel proteins (e.g., at the extreme C termini of some P5 molecules, following an extended stretch of amino acids predicted to be intracellular [M. Mullins, unpublished data]). However, significant limitations exist for even the most robust predictive tools for modeling protein structure. Definitive information as to the membrane topology of P2 and P5 in *H. parasuis* requires direct experimental evidence, which has yet to be obtained. Nonetheless, predictions resulting from the analyses reported here provide a basis for additional investigation into the structures and functions of these proteins in *H. parasuis*.

No prior data provide evidence for more than a single copy of *ompP2* in *H. parasuis*, including the draft genome sequence of strain 29755 and the completed sequence for strain SHO165. Multiple copies are not known to occur in *H. influenzae* but have been reported in *Haemophilus ducreyi* (35). Our results suggest that four of the *H. parasuis* strains evaluated here may harbor at least two copies of *ompP2*. Although these strains segregate within one of the two major lineages of the MLST phylogeny, they do not appear to be closely related. No polymorphisms were apparent within individual strains when the sequences of numerous individually cloned PCR amplicons were compared. However, it cannot currently be assumed that multiple copies of *ompP2* occurring within a particular strain are identical, since sequence polymorphisms potentially present in primer-binding regions may have restricted PCR amplification to only one of the two loci detected by Southern blotting. Future efforts directed toward determination of the DNA sequence adjacent to the *ompP2* ORF(s) will provide

definitive assessment of the copy number and also permit selective amplification and sequencing of all copies. Compared to the ATCC type strain, 19417, preliminary quantitative-RT-PCR results failed to demonstrate significantly higher levels of *ompP2*-specific mRNA in the group of strains potentially possessing multiple gene copies (C. L. Loving, unpublished data), but as noted above, it is uncertain whether the primers used permit detection of more than a single copy. Sequence analysis and comparison of promoter regions may assist in predicting whether all copies are likely to be transcriptionally active. Multiple copies of *ompP2* could add to the repertoire of P2 variants available to some strains of *H. parasuis*.

Consistent with the findings reported here, there is no prior evidence suggesting multiple copies of *ompP5* in *H. parasuis*. *H. influenzae* similarly appears to possess only a single allele, although multiple copies do occur in some other members of the *Pasteurellaceae*. In *H. ducreyi*, a second, tandemly located and independently transcribed copy of the *ompP5* ortholog has been described that is proposed to have arisen through a duplication event (20). Two tandem, independently expressed copies, one of which may have been acquired through horizontal DNA transfer, are also present in *Pasteurella trehalosi* (8).

Although the PCR primers used here for MLST are identical to those employed in a prior study (33), our respective phylogenies cannot be directly compared because the slightly truncated sequences used in the present study do not include a few extreme 5′ and 3′ base positions previously noted to be variable among strains. Some of the positions in question lie within the primer sequences themselves, raising the possibility that, in some cases, the previously reported 5′- and 3′-terminal variable positions could inadvertently represent primer sequence rather than the sequence of the DNA template. In fact, comparison of the MLST primer sets with the corresponding targets from the recently sequenced genomes of *H. parasuis* strains 29755 and SHO165 revealed numerous mismatches in four of the seven used. Accurate phylogenies derived from MLST or other sequence data require tree construction based on sequences trimmed so as to exclude the primer-binding regions and any adjacent areas covered by sequence reads from only a single strand, as was done in the present study. Nonetheless, similar to the population structure of the strains previously evaluated (33), the *H. parasuis* strains evaluated here comprise two divergent branches of a neighbor-joining tree derived from MLST data. The previous study further noted an association between a particular branch and pathogenic isolates, but we were unable to detect such a relationship here, possibly due to the relatively limited number of strains for which reliable information related to virulence is available. Further analysis using a larger number of more fully characterized strains may help to elucidate the origins of highly pathogenic isolates.

The basis for the virulence of *H. parasuis* is not understood, and isolation of the agent from both healthy and diseased pigs suggests extreme heterogeneity among strains. Although an association has been observed between pathogenic potential and particular outer membrane protein profiles (41) or whole-cell protein profiles (32), it is not known whether P2 and/or P5 is a component of such "virulence" profiles. A possible correlation between serotype and virulence has also been noted (19), but no exclusive relationship is apparent, and many isolates are nontypeable (36). Further, it is unclear whether such characteristics merely act as markers for virulence or contribute more directly to disease. We were unable to establish a correlation between virulence and any particular P2 or P5 variant, but the likelihood that these proteins are under selective pressure suggests they may contribute to virulence. This report is the first to document extensive sequence polymorphism among *ompP2* and *ompP5* genes of *H. parasuis*, and our results additionally suggest there may be a considerable degree of antigenic variation among the corresponding proteins. Further research, including isolation and more detailed characterization of these outer membrane proteins, will assist in delineating their specific functions and possible roles in virulence.

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